Purification, characterization and comparison of glutathione *S*-transferases from black-grass (*Alopecurus myosuroides* Huds) biotypes

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Abstract: In the UK biotypes of black-grass (*Alopecurus myosuroides* Huds) showing resistance to both chlorotoluron (CTU) and aryloxyphenoxypropionate graminicides are increasingly being observed. Although the precise mechanisms involved in this resistance have yet to be identified, increased herbicide metabolism has been implicated as being involved in at least some cases of resistance. Glutathione *S*-transferases (GSTs) are a group of enzymes which have been demonstrated to metabolise herbicides in some plants, and the resistant black-grass biotype Peldon contains approximately double the GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) of susceptible biotypes. To investigate further the possible role of GSTs in herbicide resistance in black-grass, a purification procedure has been developed for these enzymes. A 27.5 kDa polypeptide possessing GST activity was purified from the susceptible biotype Herbiseed. Purification of GSTs from the resistant biotype Peldon also identified this polypeptide along with an additional 30 kDa polypeptide. An in-vitro kinetic study of both crude and purified GST extracts, and western blot analysis using antisera raised against the 27.5 kDa polypeptide, suggest that the 30 kDa polypeptide may possess GST activity, and is not a precursor of the 27.5 kDa polypeptide. These results are discussed and compared to GST profiles for other weeds and crops demonstrating herbicide resistance or tolerance.

Keywords: herbicide resistance; black-grass; *Alopecurus myosuroides*; glutathione *S*-transferases; protein purification

1 INTRODUCTION

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a group of enzymes which catalyse the nucleophilic substitution reaction of a wide variety of substrates with the tripeptide glutathione. The hydrophobic substrates are all electrophilic and are usually foreign to the organism. GSTs are found throughout the animal and plant kingdoms.[1] In animals they have been extensively studied and are implicated in many roles, including toxin and drug metabolism. Although studied to a lesser extent in plants, GSTs have been implicated in herbicide resistance,[2–6] crop tolerance to herbicides,[7–11] various stress responses, and also in secondary metabolism. Plant GSTs have recently been reviewed by Marrs.[1] GSTs have been linked to the metabolism of the herbicides alachlor, metolachlor, fluorodifen,[10] fenoxaprop,[12] dimethenamid,[11] and atrazine.[13] GSTs have been studied in maize (*Zea mays* L), soybean (*Glycine max* (L) Merr), and wheat (*Triticum aestivum* L). In maize multiple GSTs with subunit MWs of 29, 27 and 26 kDa have been identified. GSTs composed of these subunits exhibited activity towards the herbicides atrazine, alachlor, metolachlor and fluorodifen.[10] Activity against these herbicides was also detected in a variety of associated weed species.[4] In soybean, a GST containing 26 kDa subunits has been identified with activity towards metolachlor.[7] Activity against the herbicides fomesafen, acifluorfen and chlorimuron-ethyl have also been reported in this species.[9] Wheat is also reported to contain GST activity against the herbicides fenoxaprop and fluorodifen,[8] and contains a safener-induced GST with 26 kDa subunits.[11] The involvement of GSTs in the metabolism of many herbicides has generated interest in a potential role for these enzymes in herbicide resistance in weeds and crops. Herbicide resistance may arise due to an altered target site, increased metabolism or increased compartmentalisation of the herbicide away from its site of action. Thus, an inherited increase in the amount and/or activity of enzymes responsible for herbicide metabolism may lead to a weed or crop population demonstrating resistance to herbicides metabolised by the enzyme in question.

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Alopecurus myosuroides Huds (black-grass) is a major problem weed in autumn-sown cereal crops in the UK and its presence can cause reductions in both crop quality and yield.[14] This grass weed is chemically controlled using urea herbicides (eg chlorotoluron, CTU) and more recently with aryloxyphenoxypropionates (eg fenoxaprop-ethyl). However, in the early 1980s populations of black-grass were reported to be resistant to CTU[15] and this has become an increasingly observed problem, with reports of resistance in the UK, Germany, France, the Netherlands, Spain and Israel.[16,17] Cross-resistance and multiple resistance have also been observed. Cross-resistant biotypes display resistance to two or more herbicides due to a single resistance mechanism, whereas multiple-resistant biotypes possess two or more distinct resistance mechanisms.[16] Laboratory investigations have suggested that increased metabolism may be, at least in part, the mechanism responsible for resistance in black-grass, involving both cytochrome P450 monooxygenases and GSTs.[5,17] We have shown that the black-grass biotype Peldon (resistant to CTU and fenoxaprop) has approximately twice the GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) of susceptible biotypes.[3,6] This raised activity is constitutive, not requiring herbicide treatment to be expressed.[3,6] However, it is important to recognise that CDNB is only a model substrate. Indeed, not all GSTs metabolise CDNB and it is possible that isoforms that metabolise herbicides may not metabolise CDNB, and *vice versa*. Further study, utilising antisera raised to a wheat GST, has revealed that Peldon and the resistant biotype Lincs E1 may contain a polypeptide possessing GST activity which is not present in susceptible biotypes.[5] However, in a herbicide-resistant biotype of the weed *Abutilon theophrasti* Medic, raised GST activity seems to be due to elevated levels of a GST that is also present in susceptible biotypes.[2] In this study a purification scheme for GSTs from black-grass has been developed in order that their role in herbicide metabolism in this important grass weed can be further evaluated.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Black-grass seeds from plants susceptible to CTU were obtained from Herbiseed Ltd, Berkshire, UK, and seeds from plants resistant to CTU[3,18] and fenoxaprop (Milner LJ, 1998, pers comm) were collected by hand at Peldon, Essex, UK (July 1996). The Herbiseed biotype is a black-grass population grown without any herbicide application for at least seven generations (Herbiseed Ltd, Berkshire, UK, pers comm). Seeds were sown in J Arthur Bower's peat-based multi-purpose compost and watered from below. All plants were grown under glasshouse conditions, 20 °C day, 15 °C night (±5 °C), with supplementary lighting from high-pressure 250-W sodium lamps for 16 h each day. Plants were harvested at the three-leaf stage and either processed immediately or frozen in liquid nitrogen and stored at −70 °C until needed.

2.2 Glutathione *S*-transferase activity assayed toward CDNB

GST activity was extracted and assayed using an adaptation of the method of Reade *et al.*[6] Approximately 0.5 g of frozen tissue was ground to a powder in a pestle and mortar and thawed in potassium phosphate buffer (0.1 M, pH 7.0; 5 ml) containing sodium ascorbate (10 mM) and diethylenetriaminepentaacetic acid (DTPA; 5 mM). Polyvinylpolypyrrolidone (PVPP) was added at 40 g litre^{-1} prior to the addition of the extract to inhibit polyphenoloxidase activity. The extract was homogenised for 20 s using an Ultra-turrax homogeniser and centrifuged (15000 *g*, 15 min at 4 °C). The supernatant was desalted on a 10-ml Sephadex G-25 column previously equilibrated with potassium phosphate buffer (0.1 M, pH 7.0; 25 ml) containing DTPA (0.25 mM). The sample (2.5 ml) was loaded onto the column and eluted with 3.5 ml of the equilibration buffer. Samples were either used for immediate study or stored at −70 °C.

GST activity was determined using CDNB as an artificial substrate. Black-grass extract (50 µl unless otherwise stated) was added to potassium phosphate buffer (0.1 M, pH 6.5; 950 µl) containing reduced glutathione (GSH) and CDNB both at a concentration of 1 mM in a final assay volume of 1 ml. Conjugate formation was monitored spectrophotometrically at 340 nm for 4 min at 20 °C. Non-enzymic conjugate formation was determined by replacing the plant extract with an equal volume of assay buffer. Activity was calculated as nmol CDNB min^{-1} mg^{-1} total protein using the molar extinction coefficient of conjugate formed (9.6 mM^{-1} cm^{-1}). Protein content of extracts was determined by the method of Bradford.[19]

2.3 Kinetic studies

Michaelis constants for CDNB and GSH were determined from Lineweaver–Burk plots. The assays were carried out as stated above with either the crude tissue extract or purified GST fractions. The concentration of each substrate was varied independently over a range of 0.25 mM to 4 mM. Each was carried out four times from three extracts.

2.4 Purification of GSTs

Frozen tissue (32 g) was ground to a powder in a pestle and mortar and thawed in 196 ml buffer A [Tris HCl (0.1 M, pH 7.5) containing Na_2EDTA (1 mM), 2-mercaptoethanol (14 mM)] and PVPP (50 g litre^{-1}). All subsequent steps were carried out at 4 °C. The extract was homogenised for 20 s using an Ultra-turrax homogeniser, filtered through four layers of muslin and centrifuged (15000 *g*, 10 min). The supernatant was adjusted to 80% saturated ammonium sulfate and stirred for 3 h. Precipitated protein was collected by

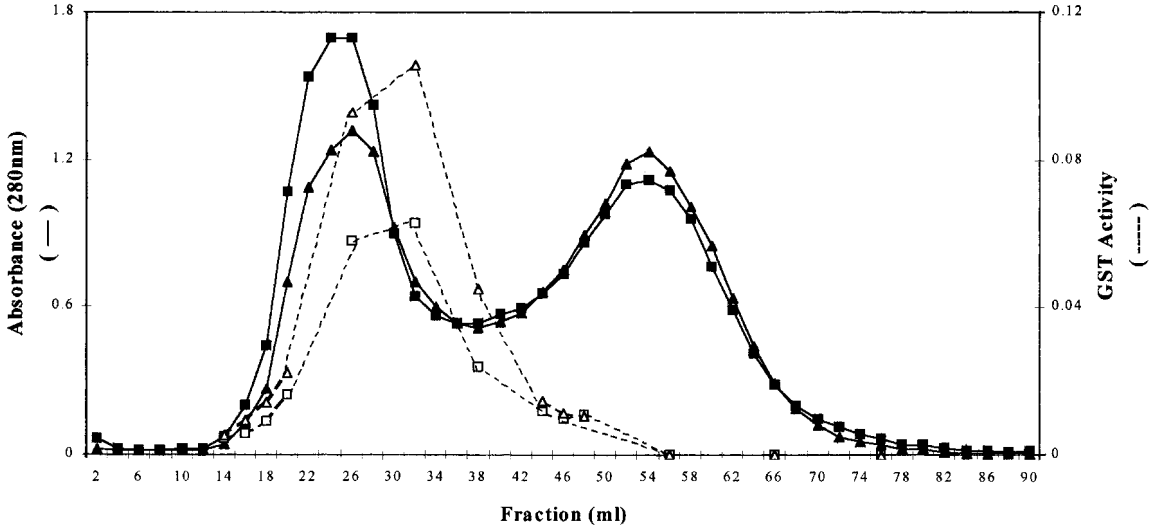

Figure 1. Behaviour of proteins extracted from (□) Herbiseed and (△) Peldon biotypes of black-grass on a 45-ml Sephadex G-75 gel filtration column. Protein (filled symbols) was monitored spectrophotometrically at 280nm. GST activity (open symbols) is expressed as absorbance change (340nm) min^{-1} 50μl^{-1} of eluted protein.

centrifugation (15000*g*, 10min), resuspended in 24ml of buffer B [Tris HCl (20mM, pH 7.8) containing 2-mercaptoethanol (14mM)] and dialysed against this buffer overnight. After clarification of the dialysed extract by centrifugation (as above) proteins were loaded onto a 4-ml Q-Sepharose column (Econo-Pac High Q, Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK), previously equilibrated with buffer B, at a flow rate of 1ml min^{-1}. The column was washed with 20 bed volumes of the buffer B, then eluted with 10 bed volumes each of the buffer B containing 0.2, 0.4, 0.6, 0.8 and 1.0M NaCl. All GST activity was found to be eluted in the 0.2M NaCl fractions. Fractions containing GST activity were pooled and buffer exchanged on a 45ml Sephadex G-75 gel filtration column, previously equilibrated with buffer C [Tris HCl (20mM, pH 7.0) containing NaCl (0.4M), dithiothreitol (1mM), DTPA (0.2mM)], at 1ml min^{-1}. Fractions containing GST activity were pooled and loaded onto a 45-ml GSH-agarose column (Sigma-Aldridge Company Ltd, Poole, Dorset, UK), previously equilibrated with buffer C, at a flow rate of 0.5ml min^{-1} followed by 50ml buffer C at 0.5ml min^{1} followed by 50ml buffer D [potassium phosphate buffer (20mM, pH 7.2) containing dithiothreitol (1mM), DTPA (0.2mM)] at 1ml min^{-1}. Proteins were eluted by reverse elution using buffer E [potassium phosphate buffer (20mM, pH 6.0) containing glutathione (10mM), DTPA (0.2mM)] at 1ml min^{-1}. Fractions containing GST activity were pooled and retained for later study. Protein concentrations in column elutants were monitored spectrophotometrically at 280nm. GST activity was assayed using CDNB as described above.

2.5 Analysis of purity

For analysis of purity of GSTs and for western blot analysis, extracts were resolved by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE),[20] using a Bio-Rad Mini-Protean II apparatus, at 200V. All resolving gels were 10% acrylamide. Proteins were either visualised using Coomassie blue protein stain[21] or transferred to 0.45μm nitrocellulose membrane for western blot analysis.[22]

2.6 Western blot analysis

Proteins immobilised on nitrocellulose (NC) membrane were detected using antisera raised against the 27.5kDa polypeptide purified from the Herbiseed biotype. After site-blocking using 30g $litre^{-1}$ milk protein, the NC was incubated with appropriate dilutions of the primary antisera, washed, then incubated with secondary antisera (horseradish peroxidase-labelled goat-anti-rabbit immunoglobulin. DAKO Ltd, High Wycombe, Bucks, UK). After a second wash step, protein-antibody conjugates were visualised using 1-chloro-4-naphthol as a substrate for the peroxidase. The use of pre-stained molecular weight protein standards (Sigma-Aldridge Company Ltd, Poole, Dorset, UK) enabled the molecular weight of polypeptides to be determined.

3 RESULTS

3.1 Purification of GSTs from black-grass biotypes susceptible and resistant to herbicides

A procedure was developed to purify GSTs from both black-grass biotypes. After tissue disruption and ammonium sulfate precipitation, proteins were resolubilised by dialysis. Initial separation was carried out by anion exchange on a 4-ml High-Q column (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK). For both biotypes one peak of GST activity was eluted from this column with 0.2M NaCl. No further GST activity could be eluted by increasing the NaCl concentration. The second column purification

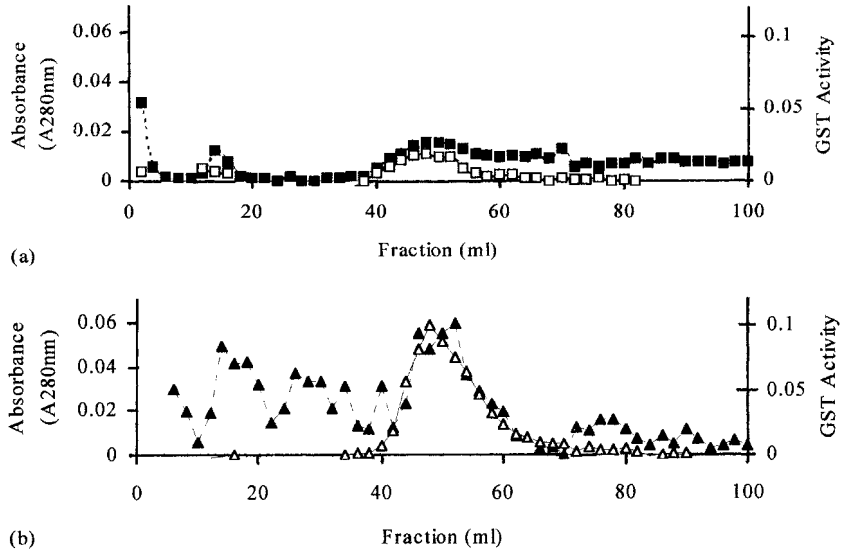

Figure 2. Behaviour of proteins extracted from (a) (□) Herbiseed and (b) (△) Peldon biotypes of black-grass on a 45-ml GSH-agarose affinity column. Protein (filled symbols) was monitored spectrophotometrically at 280 nm. GST activity (open symbols) is expressed as absorbance change (340 nm) min^{-1} 100 μl^{-1} of eluted protein.

step utilised a 45-ml Sephadex G-75 gel filtration column. This allowed a buffer exchange to be carried out prior to affinity chromatography. Both biotypes gave one peak of GST activity with this column. These peaks were associated with the first of two peaks of protein eluted from this column (Fig 1).

Final purification was achieved using a 45-ml GSH-agarose affinity column. The majority of protein did not bind to this column and was eluted during loading and the subsequent wash stages. Some GST activity was eluted at this stage, either because the column was overloaded or because binding conditions did not favour a particular GST isoenzyme. Bound proteins were eluted by combining 10 mM GSH with a change in column conditions to pH 6.0. The column was reverse-eluted in order to tighten the eluted peaks. Elution resulted in one peak of GST activity from both biotypes (Fig 2). Purification data for each biotype is given in Table 1, which demonstrates that greater GST activity resulted from the Peldon biotype.

GST-containing fractions were pooled and analysed for purity by SDS-PAGE (see Fig 3). Herbiseed (susceptible) samples revealed one, 27.5 kDa, polypeptide. Peldon extracts revealed two polypeptides, one 27.5 kDa and one 30 kDa. Similar amounts of starting material were used for extraction and the 27.5 kDa polypeptide appeared to be present at greater amounts in the Peldon extracts. These polypeptide profiles compare well with those previously detected in crude extracts of black-grass using anti-wheat GST antisera.[5] Purification of these polypeptides now allows GST activity to be ascribed to the 27.5 kDa polypeptide and suggests that the higher GST activity in Peldon may be due, at least in part, to the additional presence of the 30 kDa polypeptide. However, it remains to be demonstrated that the 30 kDa polypeptide is a subunit contributing to GST activity.

For the herbiseed biotype approximately 75% of GST activity did not bind to the affinity column and was eluted during the loading/washing steps, the remaining 25% being eluted by the glutathione. With Peldon biotype, approximately 25% did not bind, and 75% was retained and eluted with glutathione. This may suggest that the conditions used favour binding of the 30 kDa polypeptide, although Fig 3 clearly indicates that both the presence of the 30 kDa

Table 1. Purification tables for GST activities from (A) Herbiseed and (B) Peldon black-grass biotypes. Data are from purification from equal amounts of black-grass tissue

	Volume (ml)	*Total activity ($\mu mol\ min^{-1}$)*	*Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$ total protein)*	*Purification*	*Yield*
(A) Crude extract	40	2.968	0.040	1.0	100
Post-Q Sepharose column	12	1.993	0.096	2.4	67
Post-G-75 Sephadex column	26	1.508	0.056	1.4	51
Post-GSH-agarose column	18	0.364	3.181	79.5	12
(B) Crude extract	37.5	4.790	0.071	1.0	100
Post-Q Sepharose column	12	3.396	0.187	2.6	71
Post-G-75 Sephadex column	32	3.107	0.155	2.2	65
Post-GSH-agarose column	26	2.329	5.973	84.1	49

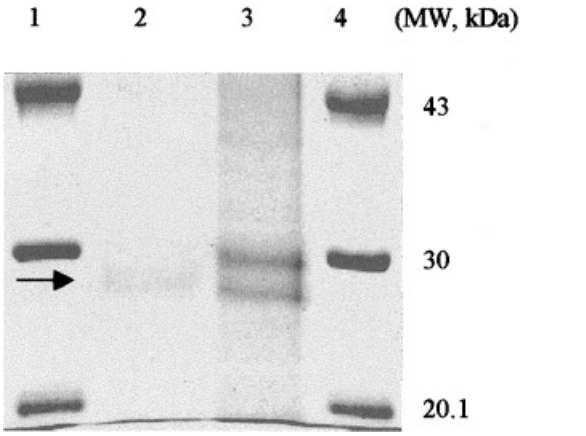

Figure 3. SDS-PAGE analysis of GSTs purified from Herbiseed (susceptible) and Peldon (resistant) biotypes. Lane 1=molecular weight markers; lane 2=Herbiseed; lane 3=Peldon; lane 4=molecular weight markers. Loadings for lanes 2 and 3 represent post-affinity column polypeptides from equal amounts of black-grass tissue.

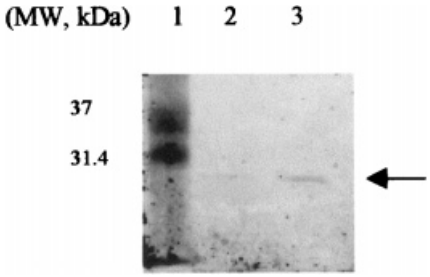

Figure 4. Western blot analysis of crude extracts of Herbiseed (susceptible) and Peldon (resistant) biotypes probed with antisera raised against purified Herbiseed GST. Lane 1=molecular weight markers, lane 2=Herbiseed, lane 3=Peldon.

polypeptide and increased amounts of the 27.5 kDa polypeptide account for the higher protein levels in post-affinity column Peldon extracts. Hence, it must be considered that the non-binding GST activity may be due to further, as yet unidentified, GST subunits and that levels of these may differ between Herbiseed and Peldon.

3.2 Western blot analysis

Antisera raised against the 27.5 kDa polypeptide were found to have a very low titre. However, western blot analysis of crude extracts of Herbiseed and Peldon did reveal that the antisera detected one, 25.7 kDa, polypeptide in each sample (Fig 4). This polypeptide is identified as being the 27.5 kDa polypeptide with GST activity purified and used to raise the original antisera. The difference in molecular mass is within the error ranges quoted for SDS-PAGE molecular mass determinations.[21] Detection of this polypeptide was poor and made it difficult to assess whether there were higher amounts of this polypeptide in the resistant biotype. Unlike previous studies of black-grass extracts utilising anti-GST antisera,[5] this study failed to identify extra polypeptides in extracts from resistant biotypes. Previous studies have used anti-wheat GST antisera and failure to detect using anti-black-grass GST antisera may demonstrate an epitopic difference between the two black-grass polypeptides. However, the low titre of the antisera and the poor quality of the blot must be taken into account when considering the absence of the extra bands on the western blot.

3.3 Enzyme kinetics

Michaelis constant data for GSTs from susceptible and resistant black-grass plants are given in Table 2. Comparison of V_{max} data for crude and purified GST samples suggests a purification of approximately 65 times. This is backed up by analysis of samples taken throughout the extraction processes (Table 1). This purification value appears low when it is considered that a single polypeptide is present in the final GST pools from susceptible plants. The purified GSTs were found to be very unstable in the post-affinity column samples. All activity was lost from Herbiseed samples within 24 h of purification. This loss of activity may explain the low purification value. It is possible that the higher GSH K_m values in purified samples, indicating a lower affinity of enzyme for substrate, may also be due to instability of the purified GSTs *in vitro*. It also must be considered that, as GSTs are dimeric, the kinetic data may be the result of more than one GST isoform. Hence, care must be taken when interpreting the results. However, if increased GST activity was due to increased abundance of a single isoform, it would be expected that the K_m values for crude extracts of susceptible and resistant black-grass would be similar. As this is not the case for K_m(CDNB) (Table 2) it implies that the increased GST activity in extracts from the resistant biotype may be due to the presence of a different GST isoform.

Table 2. Michaelis constants for crude and purified GSTs from Herbiseed and Peldon black-grass biotypes

	Crude GST extract				*Purified GST fraction*			
	GSH K_m (mM)	*GSH V_{max}*[a]	*CDNB K_m (mM)*	*CDNB V_{max}*[a]	*GSH K_m (mM)*	*GSH V_{max}*[a]	*CDNB K_m (mM)*	*CDNB V_{max}*[a]
Herbiseed (susceptible)	0.64	64	3.67	149	1.07	5270	1.56	6990
Peldon (resistant)	0.66	164	2.57	308	1.15	4460	2.32	10345

[a] GST activity as nmol $min^{-1} mg^{-1}$ total protein.

4 DISCUSSION

Investigation of GSTs in relation to herbicide metabolism has until now generally focused on crops rather than the associated weed species. In order to investigate the roles that GSTs play in herbicide resistance in these weed species, study and classification of their GSTs is necessary.

GSTs have been studied in various weeds including black-grass. The herbicide-resistant black-grass biotype Peldon has been demonstrated to have approximately double the GST activity of susceptible biotypes.[6] This increased activity is constitutive, not requiring induction by herbicides. Treatment of susceptible biotypes with herbicide safeners resulted in an increase in GST activities which was not found with the Peldon biotype. Investigation of resistant and susceptible black-grass biotypes with antisera raised against wheat GSTs revealed one 25 kDa polypeptide in susceptible biotypes, which was present at higher amounts in resistant biotypes along with two additional polypeptides (27 and 28 kDa).[5]

Purification of GSTs from the susceptible biotype Herbiseed revealed one, 27.5 kDa, polypeptide possessing GST activity. This polypeptide was also present in the Peldon biotype along with a 30 kDa polypeptide, as reported with anti-wheat GST antisera studies.[5] The smaller polypeptide appeared to be present at higher amounts in the resistant population (Fig 3). Antisera raised against the 27.5 kDa polypeptide purified from the Herbiseed (susceptible) biotype detected the 27.5 kDa polypeptide in Peldon extracts indicating this to be the same protein (Fig 4). Unlike previous studies using antisera raised to other species GSTs, the antisera raised against the black-grass polypeptide did not detect the 30 kDa polypeptide in Peldon extracts. This indicates that the larger polypeptide is a distinct protein rather than a pre-processed version of the 27.5 kDa polypeptide. It also suggests that the 27.5 kDa polypeptide is not a breakdown product of the 30 kDa polypeptide. Increased GST activity in the Peldon biotype may be a result of increased amounts of the 27.5 kDa polypeptide or the combination of this with the presence of the 30 kDa polypeptide, although GST activity has yet to be attributed to the larger polypeptide. If the raised activity were due to increased amounts of the 27.5 kDa polypeptide then it would be expected that, whereas V_{max} would be higher in Peldon extracts, K_m would not alter. Studies with crude extracts revealed K_m values similar for GSH, but different for CDNB (Table 2). The K_m values for CDNB with purified extracts are also different for Herbiseed and Peldon (although these data must be treated with caution due to the observed loss of GST activity in purified extracts). This suggests that the higher GST activity in Peldon is due, at least to some extent, to a different GST isoform. It would seem likely from comparison with other weed and crop GST profiles that both increase in the amount of the 27.5 kDa polypeptide and the presence of the 30 kDa polypeptide, suggested to be a GST subunit, contribute to the higher GST activities in the resistant biotype Peldon.

In conclusion, a purification scheme for a GST from black-grass has been developed. A 27.5 kDa polypeptide with GST activity has been identified in herbicide-susceptible black-grass. Herbicide-resistant black-grass yielded this polypeptide in greater amounts and in addition, a 30 kDa polypeptide. Although GST activity has not been assigned to this 30 kDa polypeptide, kinetic study of the crude GST extracts has identified a difference in K_m(CDNB) values between susceptible and resistant tissues, suggesting that the raised GST activities observed in resistant tissue are due, at least in part, to a separate GST as opposed to only raised levels of the 27.5 kDa polypeptide. Antisera study suggests that the 30 kDa polypeptide is not a precursor of the 27.5 kDa polypeptide. It is postulated that this polypeptide is a separate GST subunit that may play a role in herbicide resistance in the Peldon biotype. On the other hand, it is clear that the role of GST isoforms remains to be established, and that an unequivocal demonstration of activity against herbicide substrates will need to be demonstrated.

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